

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 38/00, C07K 14/00	A1	(11) International Publication Number: WO 00/66137 (43) International Publication Date: 9 November 2000 (09.11.00)
(21) International Application Number: PCT/US00/11225 (22) International Filing Date: 27 April 2000 (27.04.00) (30) Priority Data: 09/302,765 30 April 1999 (30.04.99) US (71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventor: TAKAHASHI, Hiroshi; Apartment 1701, 4 Longfel- low Place, Boston, MA 02114 (US). (74) Agent: MEIKLEJOHN, Anita, L.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: ASIALOCYTOTOKINES AND TREATMENT OF LIVER DISEASE (57) Abstract The invention features methods for treating liver disease (e.g., viral hepatitis) by administering an asialocytokine (e.g., asialointerferon). The invention also includes methods of targeting a glycoprotein to a hepatocyte and a composition containing an asialocytokine.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

ASIALOCYTOTOKINES AND TREATMENT OF LIVER DISEASE

5

Cross Reference to Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/721,828, filed September 27, 1996, now pending; which claims priority from U.S. Provisional Application Serial No. 60/004,357, filed September 27, 1995, now abandoned.

10

Statement as to Federally Sponsored Research

This invention was made with government support under National Institutes of Health grants CA57584 and NIDDK4331. The Government has certain right in the invention.

15

Background of the Invention

Hepatitis B virus (HBV) infection is a worldwide health problem. It causes a wide spectrum of pathologies ranging from inapparent infection to fatal hepatocellular diseases (Tiollais et al., Nature 317:489, 1985). The HBV virion is composed of an envelope, which carries the hepatitis B surface antigen (HBsAg), and a nucleocapsid. The nucleocapsid encloses a circular, partially double-stranded 3.2 kb DNA, which replicates via a RNA intermediate. The nucleocapsid is formed by the hepatitis B core antigen. When virions are present in the blood, an additional soluble antigen related to the nucleocapsid, the hepatitis B e antigen (HBeAg), is generally detected in the serum. Several studies have suggested that HBV is not directly hepatocytopathic and that host immune response to viral antigens presented on the surface of infected liver cells may play an important role in pathogenesis (Mondelli et al., J. Immunol. 129:2773, 1982; Mondelli et al., Arch. Pathol. Lab. Med. 112:489, 1988; Chisari et al., Microb. Pathog. 6:311, 1989).

20

25

30

The lack of suitable animal models for hepatitis B has hindered

understanding of the molecular mechanisms responsible for hepatocyte death and viral clearance (Ochiya et al., Proc. Natl. Acad. Sci. USA 86:1875, 1989; Gripon et al., J. Virol. 62:4136, 1988). Germ-line transgenic mouse models have been produced to investigate the pathogenesis of HBV infection, but these animals are immunologically tolerant to HBV antigens and do not spontaneously develop hepatitis (Moriyama et al., Science 248:361, 1990). Hepatitis must be induced in these animals by a complicated, multi-step process involving, e.g., priming lymphocytes with HBV proteins in syngeneic animals and adoptive transfer of the primed cells *in vivo* (Moriyama et al., supra; Ando et al., J. Exp. Med. 178:1541, 1993).

Summary of the Invention

The invention is based, in part, on the discovery that asialo-interferon- β (asialo-IFN- β) effectively inhibits hepatitis B virus (HBV) replication in hepatocytes. Surprisingly, this level of inhibition achieved was higher than that achieved with native interferon- β , a result contrary to the previous understanding that asialo-interferon- β is less effective than native interferon- β in inhibiting virus replication in hepatocytes.

Accordingly, the invention features a method of treating viral hepatitis in a mammal (e.g., a human) by administering to the mammal a composition which includes a therapeutic amount of an asialo-interferon (e.g., asialo-IFN-alpha, asialo-IFN- β , or asialo-IFN-gamma). The method optionally includes a step of confirming that the mammal has viral hepatitis, e.g., hepatitis caused by hepatitis B virus infection or hepatitis C virus infection.

The optional confirming step can include measuring the level of hepatitis virus replication in the mammal. The level of virus replication can be measured by any means well known in the art, including hepatitis virus-specific polymerase chain reaction or by detecting hepatitis viral antigen (hepatitis B virus e antigen) in a

bodily fluid (e.g., blood) of the mammal.

The composition can be administered via any suitable route, including subcutaneously, intramuscularly, intraarterially, or intravenously. In addition, the therapeutic amount can be about, e.g., 0.02 to 200 $\mu\text{g/kg}$ body weight/day, 30 to 75
5 $\mu\text{g/kg}$ body weight/day, or alternatively, 10,000 to 200,000 IU/kg body weight/day.

The composition can further include a pharmaceutically acceptable excipient, such as dextrose, an albumin, sodium chloride, a sodium phosphate, or water.

In another aspect, the invention features a method of targeting a
10 glycoprotein to a hepatocyte by providing an asialoglycoprotein produced by removing sialic acid residues from a glycoprotein; and contacting the asialoglycoprotein with the hepatocyte. Among the glycoproteins that can be targeted to hepatocytes in this manner are IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, erythropoietin, fibroblast growth factor,
15 granulocyte-macrophage colony stimulating factor (GM-CSF), gamma interferon, tumor necrosis factor- β , leukemia inhibitory factor, macrophage colony stimulating factor (M-CSF), macrophage migration inhibitory factor, nerve growth factor, osteostatin M, platelet-derived growth factor, stem cell factor, thrombopoietin, vascular endothelia growth factor, or hepatocyte growth factor. The hepatocyte to be
20 targeted can be within a liver, and the liver can be within a mammal (e.g., a human). The asialoglycoprotein can be contacted with the hepatocyte by intravenous, intraarterial, subcutaneous, or intramuscular injection of the asialoglycoprotein into the mammal.

The invention also features a composition including asialoglycoprotein
25 produced by removing sialic acid residues from a glycoprotein, where the glycoprotein is interleukin-1 (IL-1), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, erythropoietin, fibroblast growth factor, granulocyte-macrophage colony stimulating factor (GM-CSF), gamma interferon, tumor necrosis

factor- β , leukemia inhibitory factor (LIF), macrophage colony stimulating factor (G-CSF), macrophage migration inhibitory factor, nerve growth factor, osteostatin M, platelet-derived growth factor, stem cell factor, thrombopoietin, vascular endothelia growth factor, or hepatocyte growth factor. The invention also features a method for
5 preparing a medicament, e.g., a medicament for treatment of a liver disorder (e.g., hepatitis) by admixing an asialoglycoprotein with a pharmaceutically acceptable carrier. The composition can further include a pharmaceutically acceptable excipient.

The methods and compositions of the invention can be used to deliver a
10 glycoprotein to hepatocytes, including the delivery of IFN, in its asialo form, to treat viral hepatitis. These asialo glycoproteins are expected to have a higher specific activity in the liver than the native glycoproteins when administered to a patient. The use of other glycoprotein cytokines for treatment of specific diseases are also well known. For example, asialo-IL-1 can be used to treat anemia, as can asialo-IL-
15 3, asialo-IL-6, and asialo-erythropoietin. Asialo-IL-7 and asialo-IL-10 can be used to stimulate the growth and differentiation of T cells. Asialo-IL-12 can be used to activate natural killer cells. Asialo-GM-CSF and asialo-G-CSF can be used to activate and proliferate macrophages.

In addition, asialo-IL-1 can be used to treat fulminant or subacute
20 hepatitis. Asialo-IL-3 can be used to treat pancytopenia. Asialo-IL-6 can be used to treat fulminant hepatitis or acute exacerbation of chronic active hepatitis, or can be used to produce acute phase reactants for host defense. Asialo-IL-10 can be used to treat autoimmune hepatitis or primary biliary cirrhosis. Asialo-IL-12 can be used to treat hepatocellular carcinoma, hepatic metastatic tumors, HBV infection, hepatitis C
25 virus infection, AIDS, or parasitic infections. Asialo-GM-CSF can be used to treat malignant tumors or leukemia. Hepatocyte growth factor can be used to treat hepatic cirrhosis, liver fibrosis, or chronic hepatitis.

Thus, the invention also includes a method of treating anemia in a

mammal by providing a composition having a therapeutic amount of an asialoglycoprotein, the asialoglycoprotein being produced by removing sialic acid residues from a glycoprotein (e.g., IL-1, IL-2, or erythropoietin; and by administering to the mammal the composition.

5 Also included in the invention is a method of stimulating growth or differentiation of a T cell by providing an asialoglycoprotein produced by removing sialic acid residues from a glycoprotein (e.g., IL-7 or IL-10); and contacting the T cell with the asialoglycoprotein. The T cell can be within a mammal, and the contacting step can include administering to the mammal a composition comprising
10 the asialoglycoprotein.

 The invention also includes a method of stimulating growth or differentiation of a macrophage by providing an asialoglycoprotein produced by removing sialic acid residues from a glycoprotein (e.g., GM-CSF or M-CSF); and contacting the macrophage with the asialoglycoprotein. The macrophage can be
15 within a mammal, and the contacting step can include administering to the mammal a composition comprising the asialoglycoprotein.

 In yet another aspect, the invention includes a method of treating hepatitis in a mammal (e.g., a human) by providing an asialoglycoprotein (e.g., IL-1, IL-6, IL-10, IL-12, or hepatocyte growth factor) produced by removing sialic acid
20 residues from a glycoprotein; and administering to the mammal a therapeutic amount of the asialoglycoprotein

Brief Description of the Drawings

 Fig. 1 is a graph depicting the results of an analysis of serum HBeAg
25 level (open symbols) and anti-HBe antibody titer (closed symbols) in rats transfected with pHBV-HTD (circles) or pGEM-7Zf(+) (triangles). The relative concentrations of HBeAg and anti-HBe are given by A_{492} and percent inhibition, respectively, as described herein. Specimens whose A_{492} value is equal to or greater than the cutoff

value of 0.065 (mean of the negative control plus the factor 0.06) are considered to be positive for HBeAg, and those with a percent inhibition value equal to or greater than 50% are considered to be positive for anti-HBe.

Fig. 2 is a graph illustrating serum GPT levels in normal CD rats (□) and
5 in athymic *nude* rats (!) transfected with pHBV-HTD.

Fig. 3 is a schematic illustration of the structure of natural human IFN- α . Also illustrated are the cleavage sites of typical biantennary complex-type sugar chains of natural human IFN- α by neuraminidase. Abbreviations: Fuc, fucose; GlcNAc, N-acetylglucosamine; Man, mannose; Gal, galactose; NeuAc, N-
10 acetylneuraminic acid.

Fig. 4 is a graph of a standard curve for the quantification of HBV DNA by radioactive PCR using [α - ^{33}P]-dCTP.

Fig. 5 is a graph of HBV copy number in a culture of HBV DNA-transfected Hep G2 cells versus interferon concentration in the culture media. HBV-transfected Hep G2 cells were treated with human natural IFN-alpha, human natural
15 IFN- β , or asialo-IFN- β (at 10, 100, or 1000 IU/ml) every 24 hours for 48 hours. The reduced production of HBV in the culture supernatant of transfected Hep G2 cells is shown by the reduction in copy number of HBV DNA-containing virions that are present in one milliliter of the culture supernatant. Results are the mean plus or
20 minus one standard deviation (SD) of values obtained in triplicate experiments.

Fig. 6 is a graph of OD₅₉₀ in a cell viability assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) versus time. Hep G2 cells were treated with 1000 IU/ml of conventional IFN-alpha, IFN- β , or asialo-IFN- β every 24 hours for 72 hours. Results are the mean plus or minus SD of values
25 obtained in triplicate experiments.

Fig. 7 is a bar graph of HBV copy number in untreated ASGP receptor-negative liver cells or the same cells treated with IFN-alpha, IFN- β , or asialo-IFN- β . SK-HEP-1 cells were treated with 1000 IU/ml of cytokine every 24 hours for 48

hours. Results are the mean plus or minus SD of values obtained in triplicate assays.

Fig. 8 is a bar graph of HBV copy number in cells treated with various concentrations of asialo-IFN- β and/or asialofetuin, a competitor for the ASGP receptor. HBV DNA-transfected Hep G2 cells were treated every 24 hours for 48
5 hours with 100 IU/ml of asialo-IFN- β in the presence of various concentrations of asialofetuin (0-1.0 micromolar). Results are the mean plus or minus SD of values obtained in triplicate experiments.

Fig. 9 is bar graph of HBV copy number in cell cultures treated with asialo-IFN- β , non-specific mouse IgG1/kappa, or a mouse antibody which
10 neutralizes human IFN- β (B-02, IgG1/kappa, Japan Immuno-Monitoring Center, Inc., Tokyo, Japan). HBV DNA-transfected Hep G2 cells were treated every 24 hours for 48 hours with 100 IU/ml of asialo-IFN- β in the presence of one microgram/ml of B-02 antibody or non-specific mouse antibody. Results are the mean plus or minus SD of values obtained in triplicate experiments.

15 Fig. 10 is a plot of the relative change in serum HBV virion levels in HBV-transfected mice for various treatments.

Fig. 11 is a graph of 2-5A synthetase activity in a cell culture versus number of hours of INF treatment. The open circles represent the level of 2-5A synthetase during native IFN- β treatment. The closed circles represent the level of 2-
20 5A synthetase during asialo-IFN- β treatment.

Detailed Description of the Invention

The invention relates to the use of asialo-interferon- β to treat viral hepatitis. It is believed that asialo-interferon- β is more effective than native
25 interferon- β in the treatment of viral hepatitis because of the prevalence of asialoglycoprotein receptors on hepatocytes.

Since the carbohydrate chain of IFN- α has an extended conformation and is linked to IFN- β at some distance from the portion of IFN- β that interacts with the

IFN- α/β receptor (Karpusas et al., Proc. Natl. Acad. Sci USA 94:11813-11818, 1997), asialo-IFN- α may be able to associate with the asialoglycoprotein and IFN receptors simultaneously. This is so because the portion of the asialo-IFN- β that binds to the asialoglycoprotein receptor is located at the end of a carbohydrate chain, which has an extended conformation, and thus is spatially separated from the portion of the asialo-IFN- β that binds to the IFN- α/β receptor. Therefore, the asialo-carbohydrate moiety in the asialo-IFN- β molecule might not interfere with binding of the asialo-IFN- β to the IFN- α/β receptor.

In addition, the binding of asialo-IFN- β to the asialoglycoprotein receptor may increase the concentration of asialo-IFN- β in the vicinity of the IFN- α/β receptor, thereby facilitating binding to the IFN- α/β receptor and activation of the IFN- α/β signalling pathway. Alternatively, asialo-IFN- α may bind first to the ASGP receptor, a low affinity receptor having a dissociation constant (K_d) of approximately 10^{-6} for biantennary and approximately 10^{-8} to 10^{-9} M for triantennary galactose-terminal oligosaccharides (Lee et al., J. Biol. Chem. 258:199-202, 1983). Biantennary asialo-IFN- β bound to the asialoglycoprotein receptor may readily transfer to the IFN- α/β , which has a higher affinity ($K_d = 10^{-10}$ to 10^{-12}). Thus, it may be desirable to administer asialo-IFN- β compositions that consist predominantly of biantennary complexes rather than triantennary complexes.

The human asialo-IFN- β produced in human fibroblasts as described herein contain about 82% biantennary galactose-terminal oligosaccharides and about 18% triantennary galactose-terminal oligosaccharides. Various methods are known for creating IFN- β having a higher or lower proportion of biantennary complexes. For example, IFN produced in fibroblasts cells has a higher proportion of biantennary complexes than IFN produced in CHO cells.

IFN receptor binding is essential for IFN- α and IFN- β to elicit their antiviral activities. Although the activation of cell surface receptors by these IFNs does not require receptor internalization, binding of IFN- α or IFN- β to their

intracellular receptors may trigger IFN signaling. For example, it appears that autocrine IFN- α or INF- α does not need to reach the cell surface to exert activity. Further, IFN- α incorporated into liposomes can produce significantly greater activity than free IFN- α , probably due to delivery of this cytokine to intracellular compartments. Therefore, asialo-IFN- α may be more active than native IFN- β by its ability to activate an intracellular reservoir of IFN receptors.

Previous methods for targeting drugs (e.g., polypeptide drugs) to hepatocytes have included conjugating the drug to moieties that bind to the asialoglycoprotein receptor. See, e.g., WO 95/18636; WO 91/22310; and Nishikawa et al., Proceed. Intern. Symp. Control. Rel. Bioact. Mater. 22:502-503, 1995. These procedures can substantially increase the size of the final drug molecule. In contrast, preparation of asialo-IFN by removal of sialic acid decreases the size of the IFN molecule, thereby increasing the ability of IFN to infiltrate liver tissue *in vivo*. This is so because exchange of substances between the blood and liver parenchyma take place at specialized capillaries called Asinusoids@ that contain circular fenestrae or pores. The diameter of fenestrae ranges from 80 to 150 nm. Thus, drugs molecules whose mode of action requires passage to the liver must possess a smaller general diameter than that of the fenestrae.

Previous methods of targeting proteins to hepatocytes have involved chemical conjugation of the protein to natural asialoglycoproteins. The formation of large, multimeric macromolecules, however, may limit their accessibility to the liver *in vivo* because of their inability to pass through the fenestrae. Since asialo-IFNs, as defined and described herein, are prepared by removing sialic acids from glycosylated IFNs using neuraminidase, this modification of carbohydrate side chain does not increase the molecular size of IFNs and thus allows the IFN access to hepatocytes in the liver. As defined herein, an asialoglycoprotein (e.g., an asialo-IFN such as asialo-IFN- β) is a protein having at least one N-linked or O-linked carbohydrate group which is free of a terminal sialic acid residue.

The examples described below establish a convenient small animal model of viral hepatitis produced by transfecting human hepatitis viral DNA into mouse hepatocytes. Also described below is the use of asialo-IFN- β in reducing hepatitis viral replication *in vitro* and *in vivo*.

5 Preparation of a Rat Model of Viral Hepatitis

A head-to-tail homodimer of cloned HBV (pHBV-HTD), adw sub-type, was constructed and inserted into the EcoRI site of the pGEM-72f(+) vector (Promega, Madison WI) as described by Blum et al., J. Virol. 65:1836, 1991 and Blum et al., Hepatology 14:56, 1991. (The cloning of the adw subtype is described
10 in Valenzuela et al., The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes, in Animals Virus Genetics, Fields et al., eds., Academic Press, New York, 1980, p.57.) In some cases, a head-to-tail heterodimer of HBV, adwR9, a replication-competent HBV construct similar to pHBV-HTD was used for transfection (Blum et al., J. Virol. 65:1836, 1991 and Blum et al.,
15 Hepatology 14:56, 1991).

A two-thirds hepatectomy was performed according to the Higgins-Anderson method (Higgins et al., Arch. Pathol. 12:186, 1931) 24 h before *in vivo* transfection, since hepatectomy increases the expression of the HBV transgene. The cloned HBV constructs were directly delivered into rat livers *in vivo* by using a
20 membrane fusion-promoting cationic lipid, dioctadecylamidoglycylspermine (Behr et al., Proc. Natl. Acad. Sci. USA 86:6982, 1989). Fifty micrograms of pHBV-HTD or pGEM-72f(+) vector was mixed with 250 μ g of the cationic lipid in 500 μ l of sterile saline (0.154 M NaCl) and allowed to form a DNA-cationic lipid complex. Animals were anesthetized with ether and injected with the DNA-cationic lipid
25 complex into the right median lobe of the liver while their portal veins were temporarily ligated by hemostatic forceps.

In addition to the above-described method, 20 μ g of pHBV-HTD (or another selected DNA molecule) can be complexed with 50 μ g asialofetuin-poly-L-

lysine and 100 μ g cationic liposome in 250 μ g HBSS and injected as described above.

HBV RNA in Rat Tissues.

To investigate the expression of HBV in transfected rats, total RNA was extracted from selected tissues of rats sacrificed 3 days after *in vivo* transfection with pHBV-HTD and amplified by RT-PCR (Chelly et al., Nature 333:859, 1988). A 659-bp PCR fragment of HBV transcript was detected in the liver but not in other tissues. In this experiment total RNA isolated from a pHBV-HTD transfected Huh-7 hepatoma cell line served as a positive control.

The level of HBV expression was measured as follows. Total RNA was extracted from the liver, heart, lung, kidney, intestine, and spleen from rats transfected with pHBV-HTD and digested with RNase-free DNase I (2 units/ μ g of RNA) (Promega) for 30 min. at 37°C. cDNAs were synthesized by extension of antisense primers with reverse transcriptase (BRL) in a mixture containing 2 μ g of total RNA. PCR of the cDNA was performed in a final volume of 100 μ l containing 2.5 mM $MgCl_2$ and 100 pmol of each primer. The amplification cycles were 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min. After 35 cycles, the PCR products were separated by electrophoresis on a 1.5% agarose gel and transferred to Hybond-N+ nylon membranes (Amersham). The Southern blot was hybridized with a ^{32}P -labeled HBV-specific restriction fragment (Aat II fragment of pHBV-HTD; 3221 bp). The primers used for amplification were located within the S open reading frame of HBV (sense primer, 5'-TGCGGGTCACCATATTCTTGGAACAAGA-3' (SEQ ID NO:1); antisense primer, 5'-AGTCTAGACTCTGCGGTATTGTGAGGATTCTTG-3' (SEQ ID NO:2), which yielded a 659-bp fragment. α -actin primers that amplified an 838-bp fragment were used as a control (sense primer, 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3'; SEQ ID NO:3); antisense primer, 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'; SEQ ID NO:4). Total RNA obtained from a human hepatocellular carcinoma cell line (Huh-7)

transfected with pHBV-HTD *in vitro* (Blum et al., Hepatology 14:56, 1991) served as a positive control for detection of HBV RNA.

HBV Virion in Rat Sera

The presence of HBV virions in serum was assessed by detecting HBV
5 DNA by PCR after DNase I treatment of rat sera and immunoprecipitation of HBV
virions with anti-HBsAg-conjugated beads. Sera were positive for virions in 18 to
21 rats. The amount of HBV detected in the serum increased during the first 3 days
after transfection as assayed by increases in PCR amplifiable material detected by
Southern analysis and then rapidly decreased and could no longer be detected 14
10 days after the transfection.

HBV virion sera was measured as follows. Sera were first treated with
20 units of DNase I (Boehringer Mannheim) per ml at 37EC for 30 min to digest
naked DNAs, such as pHBV-HTD plasmid. The HBV particles were then
immunoprecipitated from the sera by using mouse anti-HBsAg antibody (5D3
15 monoclonal antibody) (Takahashi et al., J. Immunol. Methods 112:91, 1988)
conjugated to azlactone-acrylamide copolymer beads (Pierce). HBV is
immunoprecipitated by these antibody-conjugated beads because the complete HBV
virion contains double-stranded 3.2-kb HBV DNA and carries HBsAg on its
envelope. After extensive washing, HBV DNA was released from the beads by
20 heating at 95EC for 5 min, amplified by PCR by using the same primers described
above, and hybridized to the HBV probe in a Southern analysis to confirm the
specificity of the PCR products (Liang et al., J. Clin. Invest. 84:1367, 1989).

As an alternative, PCR analysis can be performed as follows. PCR
analysis of HBV is performed in a final volume of 50 μ l with 2.5 mM MgCl₂ and 1
25 μ M of each primer. The cycles are 95EC for 30 s, 50EC for 1 min and 72EC for 1
min. After 30 cycles, the PCR products are separated by electrophoresis on 1.5%
agarose gel and transferred to Hybond-N⁺ nylon membranes (Amersham). The blot
is hybridized with a ³²P-labeled HBV-specific restriction fragment (*Aat*II fragment

of pHBV-HTD; 3221bp) for Southern analysis. The following primers, located within the core open reading frame of HBV gene, are used for the detection of HBV: sense primer, GAGAATTCAAGCCTCCAAGCTGTGCCTGG (SEQ ID NO:5); anti-sense primer, GAAAGCTTCTGCGACGCGGCGATTGAGA (SEQ ID NO:6).

- 5 These primers yield a 578 bp fragment. The following β -actin primers are used as a control: sense primer, ATCTGGCACCACACCTTCTACAATGAGCTGCG (SEQ ID NO:7); anti-sense primer, CGTCATACTCCTGCTTGCTGATCCACATCTGC (SEQ ID NO:8). These primers yield a 1045 bp fragment.

10 HBV Liver DNA

HBV DNA was detected by genomic Southern analysis of liver DNA isolated from rats transfected with adwR9. DNA bands of 7.2 kb and 3.2 kb were detected in *EcoRV*-digested genomic DNAs by hybridization with an HBV-specific probe (*Aat* II fragment of pHBV-HTD). The 7.2 kb band was also seen in the same liver DNA blots rehybridized with a vector-specific probe [pGEM-7Zf(+)] DNA digested by *Bam*HI], but the 3.2 kb band was not detectable. Since both the HBV genome (adw subtype; 3.2 kb) and the adwR9 HBV constructs (7.2 kb) have a single *EcoRV* site (Blum et al., *Hepatology* 14:56, 1991), the observed 3.2 kb band was not derived from the digested adwR9. These data confirm that HBV DNA was produced and present in an unintegrated form in the liver. In addition, the HBV DNA was detected at a similar intensity on days 1, 2, and 3, although the adwR9 plasmid DNA in the liver rapidly decreased between day 1 and day 3. Thus, the presence of 3.2 kb HBV DNA after the clearance of the adwR9 construct from the liver indicates that HBV production may have been mediated by its own replication.

- 25 Genomic Southern analysis was performed as follows. Rat livers transfected with a head-to-tail heterodimer of HBV, adwR9, were used for analysis. DNA was extracted from the livers, digested with *EcoRV*, which cuts the HBV genome and adwR9 at a single site, and separated by electrophoresis through a 1%

agarose gel. The DNA fragments were transferred to a Southern hybridization filter and the blots were first hybridized with an HBV-specific restriction fragment (*Aat* II fragment of pHBV-HTD), stripped, and then rehybridized with a vector-specific restriction fragment [pGEM-7Zf(+)] DNA digested with *Bam*HI] for Southern analysis.

Serum HBeAg and Anti-HBe Antibody Response in Transfected Rats

A representative time course of serum HBeAg level and anti-HBe antibody response in pHBV-HTD-transfected rats is shown in Figure 3. HBeAg was found in rat serum 3-7 days after liver transfection with pHBV-HTD and was followed by an increase in anti-HBe antibody titer by day 21. Neither HBeAg nor anti-HBe was found in the sera of mock-transfected rats transfected with pGEM-7Zf(+) (Fig. 1).

HBeAg and anti-HBe antibody were measured as follows. Sera were collected from tail veins of rats. The presence of HBeAg and anti-HBe antibody were determined by "sandwich" and "competitive-binding" techniques, respectively, using commercially available ELISA kits (Abbott). The relative concentration of HBeAg was represented by the absorbance value of specimens at 492 nm (A_{492}). The level of anti-HBe was expressed as percent inhibition calculated by using the following formula; percent inhibition = [(mean A_{492} of negative controls - A_{492} of sample)/(mean A_{492} of negative controls - A_{492} of positive controls)] X 100.

Serum Glutamic-Pyruvic Transaminase (GPT) Levels.

GPT activity in the serum was measured as an indicator of liver disease, since GPT is found primarily in the liver and released from the damaged hepatocytes. Serum GPT values were elevated in the majority of rats 2-3 weeks after HBV transfection [60 international units (IU)/l " 5IU/l at day 0 and 210 IU/l " 49 IU/l at day 21; mean " SEM, $n = 15$] (Fig. 2). No serum GPT elevation was observed in the mock-transfected rats (37 IU/l " 18 IU/l at day 0 and 30 IU/l " 12 IU/l at day 21, $n = 3$).

Liver Histology.

Liver tissues were obtained from randomly chosen rats sacrificed 3-21 days after *in vivo* transfection with the pHBV-HTD, adwR9, or pGEM-7Zf(+) construct. In the liver tissue histology of a rat 21 days after *in vivo* transfection with the pHBV-HTD construct with a serum GPT level of 483 IU/l (see Fig. 2), the parenchymal cells in the vicinity of the portal vein had disappeared and were replaced by the infiltration of lymphocytes. Other animals transfected with pHBV-HTD or adwR9 demonstrated similar histological changes. No hepatocyte death or lymphocyte infiltration was observed in the livers of mock-transfected rats.

10 *In vivo* Transfection of Athymic Nude Rats with pHBV-HTD.

To see if T lymphocytes are important for the induction of liver cell injury in the experimental animal model described herein, T-lymphocyte-deficient athymic *nude* rats were transfected with pHBV-HTD as described above. No serum GPT elevation was observed in any of these transfected animals (Fig. 2) and their livers were histologically normal. It is interesting to note that the serum level of HBV virions increased between 7 and 21 days in these *nude* rats. This finding was in contrast to the serum HBV virion levels of normal rats, which rapidly decreased by 7-14 days after the transfection.

Characterization of the Model

20 After *in vivo* transfection of clone HBV DNA according to the above described technique, HBV RNA as well as 3.2-kb HBV DNA were present in the liver, and HBV virions were detected in the blood. Most importantly, HBeAg, a serological marker of active viral replication (Brechot et al., Lancet, ii:765, 1981; Hadziyannis et al., Hepatology 3:656, 1993), was found in rat sera, and its appearance was followed by an anti-HBe antibody response. These data indicate that HBV virions were actively produced and that an immune response to HBV gene products was elicited in rats transfected with the HBV constructs. Furthermore, the liver histology in these animals demonstrated severe hepatocellular injury

characterized by hepatocyte death and lymphocyte infiltration when serum GPT values were elevated. Thus, HBV-induced pathogenesis in these transfected rats was characterized by the expression of HBV genes, the production of HBV virions, the increase of serum transaminase, and the characteristic histological findings. These pathological changes in rats transfected with HBV DNA are similar to those found in acute viral hepatitis induced by HBV in humans.

These studies used the head-to-tail dimer constructs of HBV (pHBV-HTD) and adwR9. These and other head-to-tail dimer constructs contain the HBV genome and endogenous viral enhancer/promoter elements that are sufficient for the production of complete virions in human hepatoma cell lines. (Blum et al., J. Virol. 65:1836, 1991; Blum et al., Proc Natl Acad. Sci. USA 84:1005, 1987; Sureau et al., Cell 47:37, 1986; Yaginuma et al., Proc. Natl Acad. Sci. USA 84:2678, 1987; Yasinuma et al., Proc. Natl Acad. Sci. USA 84:2678, 1989), and the *in vitro* replication of HBV has been previously demonstrated in rat liver-derived cells (Shih et al., Proc. Natl. Acad. Sci. USA 86:6323, 1989; Diot et al., J. Med. Virol. 36:93, 1992). However, there had been no previous studies to determine if the HBV gene would be expressed in rat liver *in vivo* after the direct transfection of these replication-competent constructs. The present data demonstrate the expression of HBV genes, the production of HBV particles, and the development of spontaneous hepatitis in rats after a single gene transfer *in vivo*.

In vivo effect of asialo IFN- α in athymic nude mice model of HBV.

pHBV-HTD was complexed with asialofetuin-poly-L-lysine conjugate and cationic-liposome to make virus-like particles for liver-specific transfection (infectious liposome). The infectious liposome complex was injected in to the portal vein of nude mice via spleen. Seven days after *in vivo* transfection, mice were randomly selected and treated with intraperitoneal injections of physiological saline solution (PSS), IFN- α (10,000 IU/day) or asialo IFN- α (10,000 IU/day) for seven days.

Asialofetuin-poly-L-lysine conjugate and cationic liposome were prepared as described by Trubetskay et al. (Bioconjugate Chem. 3:323, 1992) and Karl et al. (Am. J. Med. Sci. 307:138, 1994). Since asialofetuin binds asialoglycoprotein receptor on hepatocytes and cationic liposome facilitates the fusion to cell membrane and delivery of DNA into hepatocytes, pHBV-HTD was complexed with asialofetuin-poly-L-lysine conjugate and cationic-liposome in order to accomplish liver-specific transfection. In brief, 50 μ l of pHBV-HTD (400 μ g/ml of DMEM), 100 μ l of asialofetuin-poly-L-lysine conjugate (500 μ g/ml of HBSS, pH 7.4) and 100 μ l of cationic-liposome (1,000 μ g/ml of HBSS, pH 7.7) were mixed in microcentrifuge tube. After 15 minute incubation at room temperature with mixing, the [asialofetuin-poly-L-lysine]-[DNA]-[cationic lipid] complex was filtered through 0.2 μ m polycarbonate membrane filter (Poretics Corporation, CA) before transfection.

To produce human asialo-IFN- β , 20 mg of insoluble neuraminidase attached to beaded agarose (about 0.22 units, Sigma) was suspended in 1 ml distilled water in a microcentrifuge tube and allowed to hydrate briefly. The tube was then quick-spun and washed three times with 1 ml of sodium acetate buffer (pH 5.5) containing 154 mM NaCl and 9 mM calcium chloride. The gel (about 72 μ l) was then resuspended in 150 μ l of the sodium acetate buffer. Glycosylated human IFN- β (3×10^6 IU/vial, which was about 0.15 mg) was then suspended in 150 μ l of sodium acetate buffer. The gel and IFN- β were then mixed and incubated on a rotating platform at 37°C for three hours. The mixture was then transferred to a Z-spin tube having a 0.2 μ m filter. The asialo-IFN- β was then separated from the neuraminidase by quick spin. Fifty microliter aliquots were made and stored at -80°C until use.

HBV particles were produced in all nude mice by *in vivo* transfection using infectious liposomes. In nude mice treated with PBS, HBV viremia continued to the end of the treatment (14 days after the transfection). Sialylated natural IFN- α (10,000 IU/mouse/day for 7 days) did not demonstrate significant anti-viral effect. In contrast, asialo IFN- α (10,000 IU/mouse/day for 7 days) demonstrated substantial

anti-viral effect.

Effect of human asialo IFN- α on HBV transfected human hepatoma cells

Asialoglycoprotein receptor bearing hepatoma cell lines were identified using [125 I]-labeled asialo-orsomucoid (Schwartz et al., *J. Biol. Chem.* 256:8878, 5 1981). One asialoglycoprotein receptor expressing cell line Hep G2 (American Type Culture Collection; Bethesda, MD: ATCC HB8065) was selected for transfection with HBV. Hep G2 cells were transfected with pHBV-HTD as described below.

To examine the effect of asialo IFN- α and natural IFN- α on HBV-transfected HepG2 cells, 2×10^5 transfected cells were treated with either human 10 natural IFN- α (1,000 IU/ml), asialo IFN- α (1,000 IU/ml), or saline for 48 hours. Cytotoxicity was monitored using a colorimetric MTT cell proliferation assay as described by Mosmann (*J. Immunol. Meth.* 65:55, 1983).

Asialo IFN- α is More Effective than the Natural IFN- α

Both asialo IFN- α and natural IFN- α were found to reduce HBV 15 production by HBV transfected Hep G2 cells. However, asialo IFN- α was found to be more effective than natural IFN- α . Asialo IFN- α reduced HBV production more than 5-fold, compared to control cells, while natural IFN- α reduced HBV production only 1.5 to 2-fold. Moreover, asialo IFN- α reduced HBsAg production by HBV transfected HepG2 cells 26-38%, while natural IFN- α reduced HBsAG production 20 33-40%.

Table 1

	HBsAg (ng/ml)		
	0 hr	48 hr	72 hr
Saline	0.018±0.006	6.934±0.175	14.530±0.280
IFN- β (1,000 IU/ml)	0.029±0.003	2.747±0.090*	3.830±0.266**
AS-IFN- β (1,000 IU/ml)	0.042±0.008	2.618±0.093*	4.751±0.109**

NOTE: Six hours after transfection, saline, IFN- β or asialo-IFN- β was added to culture medium (0 hr). Production of HBsAg from transfected HepG2 cells was significantly inhibited by IFN- β and AS-IFN- β by 48 hr treatment (*: $P = 0.00004$ vs. saline, **: $P = 0.00004$ vs. saline and $P = 0.015$ vs. IFN- β respectively by Student's t test).

The effect of asialo IFN- α and natural IFN- α on pHBV-HTD transfected SK-HEP cells was examined. These cells lack the asialoglycoprotein receptor. For these cells, asialo IFN- α was no more effective than natural IFN- α .

The following methods were used in the experiments described in this section.

For these experiments, human asialo IFN- α was prepared from natural glycosylated IFN- α as described above. HBV virion production by HBV-transfected HepG2 cells was measured as described above. HBV surface antigen (HBsAg) was examined using a enzyme-linked immunosorbent assay (AUSZYME, Abbot Laboratories).

All cell lines were maintained in Eagle's MEM (M.A. Bioproducts; Walkersville, MD) supplemented with 10% fetal calf serum inactivated at 56°C for 30 min., 10 mM non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml

streptomycin. Cells used for *in vitro* testing were harvested from the monolayer cultures by treatment with 0.04% EDTA/versine buffer in the absence of trypsin for 5 min. at 37EC.

Cells were transfected with pHBV-HTD (described above) by the calcium phosphate method (Mol. Biol. Cell. 7:2745, 1987) using 2×10^5 cells and 3 μ g of pHBV-HTD per 300 mm plate. After transfection, 30 μ l of IFN- α (100 IU/ μ l) or asialo IFN- α (100 IU/ μ l) were applied every 6 hours for 48 hours to culture medium to a final concentration of 1,000 IU/ml. The same total volume of physiological saline was added to control cultures.

10 Asialo-interferon

Asialo interferon used to treat hepatitis B and other conditions can be produced by removing a terminal sialic residue from interferon which is glycosylated and normally has such a residue by virtue of it having been produced in a eukaryotic cell (see, e.g., U.S. Patent No. 4,184,917 and references cited therein, and Kasama et al., J. Interfer. Cyto. Res. 15:407-415, 1995). The terminal sialic residue can be removed by, for example, mild acid hydrolysis, or treatment of native glycosylated IFN with isolated and purified bacterial or viral neuraminidase as described in Drzenieck et al., Microbiol. Immunol. 59:35, 1972. Neuraminidases are readily available from Sigma Chemical Co. (St. Louis, MO) as catalog nos. N 3642, N 5146, N 7771, N 5271, N 6514, N 7885, N 2876, N 2904, N 3001, N 5631, N 2133, N 6021, N 5254, and N 4883.

Native, glycosylated interferon can be isolated from human cells, which produce it naturally, or from eukaryotic cells which have been manipulated so that they express a cloned interferon gene. Methods for natural or recombinant production of interferon are generally described in U.S. Patent Nos. 4,758,510, 4,124,702, 5,827,694, 4,680,261, 5,795,779, and 4,130,641. Alternatively, isolated and purified human interferon is available from Sigma as catalog nos. I 2396, I 2271, I 1640, and I 6507.

Animal models

The methods described above may be used to prepare rodent models of other forms of hepatitis. Thus, other variant and mutant form of the hepatitis B virus may be used in place of adw2 variant used in the above-described experiments.

- 5 Thus, the adw, adr(1), adr(2), ayr, ayw(1), ayw(2) or other variants may be used. In addition, the methods described above may also be used by those of skill in the art to prepare models of hepatitis C and hepatitis G.

Treatment with Asialo-interferon

- 10 The method described above, and other techniques known to those skilled in the art, can be used to prepare asialo forms of glycosylated cytokines. Thus, it may be possible to prepare asialo forms of interferon α_2 or other glycosylated human interferons. The asialo-interferons can be used to treat a wide range of hepatic diseases or other diseases requiring administration of interferon to cells bearing the asialoglycoprotein receptor including: hepatitis B, hepatitis C, renal cell carcinoma,
15 and hepatitis G.

- The removal of a terminal sialic acid residue may be a useful method for modifying a wide range of other proteins produced in eukaryotic cells (naturally or by expression of a recombinant gene). This modification should produce an asialo-protein which can be more readily taken up by cells bearing the asialoglycoprotein
20 receptor and is thus more effective.

Asialo-IFN- β inhibits HBV replication in vitro and in vivo and is superior to native IFN- β .

- The efficacy of asialo-IFN- α was assessed by its ability to reduce the production of intact HBV virions by Hep G2 human hepatoma cells transfected with
25 a replication-competent HBV construct carrying a head-to-tail homodimer of the entire HBV genome (pHBV-HTD; see above). This liver cell line expresses asialoglycoprotein receptor at a level similar to normal human hepatocytes (Eisenberg et al., J. Hepatol. 13:305-309, 1991; and Schwartz et al., J. Biol. Chem.

256:8878-8881, 1981).

The experiments described below establish the asialo-IFN- β is more effective in inhibiting hepatitis viral replication in hepatocytes than native IFN- β . This result concurs with the finding, also discussed below, that asialo IFN- β induced
5 2'-5' oligoadenylate synthetase, an indicator of the IFN antiviral cellular response, at a level significantly higher than that induced by native IFN- β .

For quantification of the intact virions containing HBV DNA, a radioactive polymerase chain reaction (PCR) method was used. This method involved DNase I digestion of the culture supernatant and immunoprecipitation of
10 enveloped viral particles. The quantification was validated by applying this method to a serially diluted virus DNA preparation. Fig. 4 shows a precise linear relationship (linear regression correlation coefficient; $r = 0.998$, $P < 0.001$) between the incorporation of [α - ^{33}P]-labeled dCTP into the PCR product and the known number of control HBV DNA copies. Fig. 4 also provides a standard curve for
15 calculation of copy number of HBV DNA-containing intact virions in transfection experiments.

The methods carried out in this section were performed as follows. The human hepatoma cell lines SK-HEP-1 cells and Hep G2 cells (American Type Culture Collection, Rockville, MD) were cultured in the presence of 5% CO₂ at
20 37°C in Dulbecco's Modified Eagle Medium (D-MEM) (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma Chemical Co. St. Louis, MO), 100 FM non-essential amino acids, 100 U/ml penicillin, and 100 Fg/ml streptomycin.

Human asialo-IFN- β was prepared as described above.

25 Hepatoma cells (2×10^5 cells) were cultured in 30 mm plastic dishes and transfected with 3 Fg of pHBV-HTD using a calcium phosphate mammalian cell transfection kit (5 Prime63 Prime, Inc.; Boulder, CO). Transfected cells were treated with either natural IFN- α , natural IFN- β , asialo-IFN- β , or saline (control) at 24 hrs

and 48 hrs after transfection. The culture supernatant was collected at 72 hrs post-transfection. Viability of hepatoma cells treated with IFNs was assessed by means of a dye-reduction assay with 3-[4,5-dimethylthiazol-2-ol]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.; St. Louis, MO).

5 To measure HBV production, cell culture supernatants (200 Fl) were treated with 20 U/ml of DNase I (Sigma) at 37 EC for 15 minutes to degrade any plasmid or free HBV DNA. Ten microliters of EDTA (0.5 M, pH 8.0) was then added to inactivate DNase I. The enveloped virus particles were absorbed with a high affinity monoclonal antibody specific to HBV surface antigen (Takahashi et al.,
10 Proc. Natl. Acad. Sci. USA 92:1470-1474, 1995) which was covalently conjugated to azlactone-acrylamide copolymer beads (3M EmphazeJ Biosupport Medium AB1, Pierce, Rockford, IL). After extensive washing, HBV DNA was released from the beads in 50 Fl of distilled water by heating at 95EC for 10 minutes. Quantification of HBV DNA was performed by PCR using primers
15 5'-GAGAATTCAAGCCTCCAAGCTGTGCCTTGG-3' (SEQ ID NO:5) and 5'-GAAAGCTTCTGCGACGCGCGATTGAGA-3' (SEQ ID NO:6). The PCR was carried out with a hot start using AmpliWaxJ PCR Gem (PE Applied Biosystems, Foster City, CA) in a 50 Fl of mixture containing 20 Fl of DNA sample, 2.5 mM MgCl₂ with 1 FM of two primers, 0.01 mM of dNTPs, 2.5 units of Taq DNA
20 polymerase (AmpliTaq; PE Applied Biosystems, Foster City, CA) and 10 FCi of [³³P]-dCTP with amplification cycles of 95EC for 30 s, 50EC for 1 min, and 72EC for 3 min. After 25 cycles, 10 Fl of each of the PCR products were separated by electrophoresis in 6% (w/v) polyacrylamide gels. PCR product bands were located by autoradiography and excised, and the radioactivity was measured with a liquid
25 scintillation counter (Beckman Instruments, Fullerton, CA). For quantification of HBV, a standard curve was constructed from PCRs with known quantities of 3.2 kb linearized HBV DNA. To ensure the absence of the transfecting plasmid in the DNA samples, PCRs were also carried out with the sense primer located in the *lacZ*

sequence of the pGEM-7Zf(+) vector substituted for the sense primer (SEQ ID NO:5) for the HBcAg sequence. No template contamination could be detected DNase treatment.

Seven to eight week old Balb/c athymic nude (nu/nu) mice were obtained
 5 from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Throughout the experiments, these animals were maintained under specific-pathogen-free-conditions. Mice were transfected with pHBV-HTD *in vivo* using a liver-specific transfection reagent that consists of a [asialofetuin-poly-L-lysine]-[HBV DNA]-[cationic liposome] ternary complex. The N-terminal modified poly(L-lysine) was conjugated to asialofetuin
 10 using conditions similar to those described for the conjugation of poly(L-lysine) to an antibody (Trubetskoy et al., Biochim. Biophys. Acta 1131:311-313, 1992). Fifty microliters of pHBV-HTD (400 Fg/ml in D-MEM), 100 Fl of asialofetuin-poly-L-lysine conjugate (500 Fg/ml in Hank=s balanced salt solution, pH7.4) and 100 Fl of cationic-liposome containing 65 mol% 3â[N-(N=N=

15 dimethylaminoethane)carbamyoyl]cholesterol and 35 mol% oleoylphosphatidylethanolamine (1,000 Fg/ ml in Hank=s balanced salt solution, pH7.7) were combined in a microcentrifuge tube. After a 15 minute incubation at room temperature with mixing, the ternary complex was filtered through a 0.2 Fm polycarbonate membrane filter (Poretics Corporation, CA) and injected into the
 20 portal vein of nude mice via the spleen. Seven days after *in vivo* transfection, mice were randomly selected, and their blood was sampled by periorbital bleeding. They were then treated with intraperitoneal injections of saline as placebo, mouse IFN-â or asialo-IFN-â (1,000 or 10,000 IU in 200 Fl of saline/day, respectively) for seven consecutive days.

25 To measure 2'-5' oligoadenylate (2-5A) synthetase activity, HepG2 cells were treated with 100 IU/ml of human IFN-â or asialo-IFN-â for 8, 12 or 24 hours in 24-well plastic plates. The cells were washed twice with phosphate-buffered saline, lysed in lysis buffer containing 10 mM HEPES (pH7.6), 10 mM KCl, 2 mM

magnesium acetate, 7 mM 2-mercaptoethanol, and 0.5% Nonidet P-40. The cells were then sonicated for 20 seconds and centrifuged at 15,000 x g for 15 min. The protein concentrations of cell lysates were determined by Bradford dye-binding procedure using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

- 5 All samples were frozen at B80EC until assayed. The level of 2-5A synthetase activity in Hep G2 cells was measured using radioimmunoassay kit (Eiken Immunochemical Laboratory, Tokyo, Japan) as described in Shindo et al., Hepatology 8:366-370, 1988.

- HBV-transfected Hep G2 cells were treated with asialo-IFN- α and its anti-viral effect was compared with that of conventional natural human IFN- α (Sumitomo Pharmaceutical Co., Osaka, Japan) or IFN- α (Toray Industries, Tokyo, Japan). As shown in Fig. 5, asialo-IFN- α produced a significantly greater antiviral effect than conventional IFN- α or β ($P < 0.001$; asialo-IFN- α versus conventional IFN- α or β at 10, 100 and 1,000 IU/ml by Bonferroni t tests after significant ANOVA). At the 100 IU/ml dose, the HBV copy number under asialo-IFN- β treatment was less than half of the HBV copy number under native IFN- β treatment. Thus, the asialoglycoprotein was at least twice as effective in reducing HBV replication as the native counterpart. Similar results were observed using Huh-7, another human hepatoma cell line that highly expresses asialoglycoprotein receptor.
- 20 The increased inhibition of HBV by asialo-IFN- α was not due to cytotoxic effects. No cytotoxicity was observed even at the highest dose (1,000 IU/ml, every 24 hrs for 72 hrs) that was used in these experiments when examined by the MTT dye-reduction assay (Fig. 6).

- To confirm the antiviral effect of asialo-IFN- β , 2-5A synthetase activity was measured. This IFN-induced enzyme polymerizes ATP into 2'-5'-oligoadenylates which then activate a latent endoribonuclease to degrade RNAs. Since HBV replicates via an RNA intermediates, induction of 2-5A synthetase is thought to play an important role in antiviral action of IFN through its inhibition of

protein synthesis and viral replication. As shown in Fig. 11, it was found that 2-5A synthetase activity in the asialo-IFN- β -treated Hep G2 cells increased at a level significantly higher than that of conventional IFN- β -treated Hep G2 cells ($P = 0.025$ and 0.004 ; asialo-IFN- β versus conventional IFN- β by t tests at 12 hrs and 24 hrs, respectively). Thus, augmented antiviral effect of human asialo-IFN- β was confirmed by the enhanced induction of 2-5A synthetase activity.

To determine whether or not increased drug efficacy of asialo-IFN- α was mediated by asialoglycoprotein receptor on the target cells, a competitive inhibition experiment was performed using asialofetuin as a competitive ASGP receptor inhibitor. As shown in Fig. 8, the antiviral effect of asialo-IFN- α was inhibited by asialofetuin (0.01 - 1.0 μ M) in HBV-transfected Hep G2 cells ($P < 0.01$ for all pairwise comparisons by Bonferroni t tests after significant ANOVA).

The importance of ASGP receptor was further investigated using a SK-HEP-1 human hepatoma cell line that is negative for asialoglycoprotein receptor. It was found that asialo-IFN- α did not exhibit an increased antiviral effect compared with conventional IFN- α or β in this asialoglycoprotein-negative cell line.

To examine whether the enhanced antiviral effect of asialo-IFN- α is also dependent on its binding to IFN receptor, anti-human IFN- α neutralizing antibody was used to block asialo-IFN- α binding to IFN receptor. As shown in Fig. 9, the antiviral effect of asialo-IFN- α was inhibited by this neutralizing antibody, but not by an isotype-matched non-IFN-specific mouse IgG ($P < 0.001$, anti-IFN- α versus no antibody or irrelevant mouse IgG; $P > 0.05$, no antibody versus irrelevant mouse IgG by Bonferroni t tests after significant ANOVA). This result confirmed that asialo-IFN- α exerts its antiviral effect via IFN receptor signaling.

The efficacy of human asialo-IFN- β was again tested *in vivo*. In this experiment, the specificity of PCR amplifiable HBV DNA was confirmed by Southern analysis. The serum HBV virion rapidly decreased by 14 days after HBV DNA transfection of mice treated with asialo-IFN- β ($10,000$ IU), which was in

contrast to the increase in the HBV virion levels in the placebo-treated mouse. The conventional IFN- β (10,000 IU) was unable to suppress viremia below the pretreatment level. Fig. 10 summarizes the change of serum HBV virions detected by Southern analysis of PCR products at the end of treatment (at 14 days after transfection), compared to the pretreatment value (at 7 days after transfection) in each individual athymic mouse. The conventional IFN- β (1,000 or 10,000 IU) did not demonstrate a statistically significant antiviral effect compared to the placebo ($P > 0.05$; placebo versus conventional IFN- β by Bonferroni t tests). This negative result is in contrast to asialo-IFN- β (10,000 IU), which produced a statistically significant antiviral effect ($P < 0.005$, asialo-IFN- β versus placebo or 1,000 IU of conventional IFN- β ; and $P < 0.05$, asialo-IFN- β versus 10,000 IU of conventional IFN- β by Bonferroni t tests after significant ANOVA). The viremia was also suppressed below the pretreatment level in some mice at smaller dose of asialo-IFN- β (1,000 IU), but statistically significant anti-viral effect was not observed at this dose ($P > 0.05$, versus placebo by Bonferroni t tests).

The conventional recombinant mouse IFN- α (1,000 or 10,000 IU) was unable to suppress the viremia below the pretreatment level and did not demonstrate a statistically significant antiviral effect compared to the placebo ($P > 0.05$; placebo versus conventional IFN- α by Bonferroni t tests). In contrast, asialo-IFN- α (1,000 or 10,000 IU) produced a statistically significant antiviral effect ($P < 0.001$; asialo-IFN- α versus placebo or conventional IFN- α by Bonferroni t tests after significant ANOVA), and reduced the amount of virus below the pretreatment level (Fig. 10). Of note, the virus was completely eradicated and not detectable in one of three and two of three mice at the end of treatment with 1,000 or 10,000 IU of asialo-IFN- α , respectively.

In addition, the greater effectiveness of asialo-IFN- α relative to conventional IFN- α *in vivo* was confirmed by Southern analysis of PCR products using the HBV-specific restriction fragment.

The experiment in this section demonstrated that the ASGP receptor-mediated augmentation of the antiviral effect of IFN *in vitro* and *in vivo*. A significantly greater antiviral effect was produced by asialo-IFN compared with conventional IFN- α or β (Fig. 5).

5 Compared to the IFN- α/β receptor with 100-5,000 binding sites per cell, the ASGP receptor is an abundant receptor with as many as 50,000-500,000 binding sites per hepatocyte. Enhanced efficacy of this modified IFN clearly requires binding to ASGP receptor as evidenced by competitive inhibition studies (Fig. 8). More importantly, the binding of asialo-IFN- α to IFN receptor is essential for its
10 antiviral effect (Fig. 9).

 Furthermore, 2-5A synthetase was induced by asialo-IFN- α at a level significantly higher than by conventional IFN- α , confirming the IFN receptor-mediated augmentation of anti-viral effect. These observations are consistent with the hypothesis that the binding of asialo-IFN- α to the ASGP receptor facilitates
15 signaling through an IFN- α/β receptor and augments its antiviral effect.

Use

 The animal hepatitis models of the invention can be used for immunological and molecular studies of the pathologic process of hepatitis including studies of liver cell death. Importantly, the model can be used to screen potential
20 therapeutics.

 Mutational changes or deletions in the HBV genome have been identified and are believed to be associated with the development of severe forms of hepatitis; however, this hypothesis has not been tested *in vivo* because of the lack of an appropriate model system. The cellular functions of various HBV transactivator
25 proteins and the possible involvement of these proteins in the cancer process have also not been examined in normal adult hepatocytes. This hypothesis may now be amenable to experimental evaluation using the animal hepatitis model described herein by preparing animals harboring variant or mutant or another virus. In

addition, it is now possible to develop experimental models to test the anti-viral effect of therapeutic regimens *in vivo* and to investigate the pathogenicity of other hepatotropic viruses, including hepatitis C virus.

Asialo-interferon α can be used to treat hepatitis B (or hepatitis C or
5 hepatitis G) at dosages similar to or less than used by those skilled in the art for the natural form of human interferon. Because of the greater specificity, higher effective dosages will be possible with lower toxicity. Those skilled in the art will be able to determine the proper dosage through the use of animal models and dose escalation clinical trials. Of course, the effective dosage will generally be less than for natural
10 interferon which has not been treated to remove a terminal sialic acid. Other forms of interferon can be treated similarly.

Production of other asialoglycoproteins.

Asialoglycoproteins other than IFN can be produced in a manner similar to that described above for IFN. For example, glycosylated cytokines, such as IL-5,
15 IL-6, IL-9, IL-10, IL-12, fibroblast growth factor, nerve growth factor, and platelet-derived growth factor are available from Sigma as catalog nos. I 5273, I 3268, I 3394, I 3519, I 1270, F 3133, N 4273, and P 8184, respectively. These cytokines can then be treated with neuraminidase to produce asialocytokines as described above.

20 What is claimed is:

1. A method of treating viral hepatitis in a mammal, the methods comprising administering to the mammal a composition comprising a therapeutic amount of an asialointerferon.

5 2. A method of targeting a glycoprotein to a hepatocyte, the method comprising

 providing an asialoglycoprotein produced by removing sialic acid residues from a glycoprotein; and

 contacting the asialoglycoprotein with the hepatocyte,

10 wherein the glycoprotein is selected from the group consisting of IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, erythropoietin, fibroblast growth factor, granulocyte-macrophage colony stimulating factor, gamma interferon, tumor necrosis factor- β , leukemia inhibitory factor, macrophage colony stimulating factor, macrophage migration inhibitory factor, nerve growth factor,
15 osteostatin M, platelet-derived growth factor, stem cell factor, thrombopoietin, vascular endothelia growth factor, and hepatocyte growth factor.

 3. The method of claim 2, wherein the hepatocyte is within a liver, and the liver is within a mammal.

20

 4. The method of claim 3, wherein the asialoglycoprotein is contacted with the hepatocyte by intravenous, intraarterial, subcutaneous, or intramuscular injection of the asialoglycoprotein into the mammal.

5. A composition comprising an asialoglycoprotein produced by removing sialic acid residues from a glycoprotein, wherein the glycoprotein is selected from the group consisting of IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, erythropoietin, fibroblast growth factor, granulocyte-
5 macrophage colony stimulating factor, gamma interferon, tumor necrosis factor- β , leukemia inhibitory factor, macrophage colony stimulating factor, macrophage migration inhibitory factor, nerve growth factor, osteostatin M, platelet-derived growth factor, stem cell factor, thrombopoietin, vascular endothelia growth factor, and hepatocyte growth factor.

10

6. The composition of claim 5, further comprising a pharmaceutically acceptable excipient.

7. A method of treating viral hepatitis in a mammal, the method
15 comprising confirming that the mammal has viral hepatitis, and administering to the mammal a composition comprising a therapeutic amount of an asialointerferon.

8. The method of claim 7, wherein the confirming step comprises measuring the level of hepatitis virus replication in the mammal.

20

9. The method of claim 8, wherein the level of hepatitis virus replication is measured using a hepatitis virus-specific polymerase chain reaction.

10. The method of claim 8, wherein the level of hepatitis virus
25 replication is measured by detecting hepatitis viral antigen in a bodily fluid of the mammal.

11. The method of claim 10, wherein the bodily fluid is blood.

12. The method of claim 10, wherein the hepatitis viral antigen is an e antigen of a hepatitis B virus.

5 13. The method of claim 7, wherein the viral hepatitis is caused by hepatitis B virus infection.

14. The method of claim 7, wherein the viral hepatitis is caused by hepatitis C virus infection.

10

15. The method of claim 7, wherein the composition is administered subcutaneously, intramuscularly, intraarterially, or intravenously.

16. The method of claim 15, wherein the therapeutic amount is about
15 0.02 ig to 200 ig/kg body weight/day.

17. The method of claim 16, wherein the therapeutic amount is about 30 ig to 75 ig/day.

20 18. The method of claim 15, wherein the composition further comprises a pharmaceutically acceptable excipient.

19. The method of claim 18, wherein the excipient is selected from the group consisting of dextrose, an albumin, sodium chloride, a sodium phosphate, and
25 water.

20. The method of claim 15, wherein the therapeutic amount is about 10,000 to 200,000 IU/kg body weight/day.

21. The method of claim 7, wherein the mammal is a human.

22. A method of treating anemia in a mammal, the method comprising
5 providing a composition comprising a therapeutic amount of an
asialoglycoprotein, the asialoglycoprotein being produced by removing sialic acid
residues from a glycoprotein, wherein the glycoprotein is selected from the group
consisting of IL-1, IL-3, IL-6, and erythropoietin; and
administering to the mammal the composition.

10

23. A method of stimulating growth or differentiation of a T cell, the
method comprising

providing an asialoglycoprotein produced by removing sialic acid
residues from a glycoprotein, wherein the glycoprotein is IL-7 or IL-10; and
15 contacting the T cell with the asialoglycoprotein.

24. The method of claim 23, wherein the T cell is within a mammal,
and the contacting step comprises administering to the mammal a composition
comprising the asialoglycoprotein.

20

25. A method of stimulating growth or differentiation of a macrophage,
the method comprising

providing an asialoglycoprotein produced by removing sialic acid
residues from a glycoprotein, wherein the glycoprotein is GM-CSF or M-CSF; and
25 contacting the macrophage with the asialoglycoprotein.

26. The method of claim 25, wherein the macrophage is within a mammal, and the contacting step comprises administering to the mammal a composition comprising the asialoglycoprotein.

5 27. A method of treating hepatitis in a mammal, the method comprising providing an asialoglycoprotein produced by removing sialic acid residues from a glycoprotein, wherein the glycoprotein is IL-1, IL-6, IL-10, IL-12, or hepatocyte growth factor; and
 administering to the mammal a therapeutic amount of the
10 asialoglycoprotein.

1/9

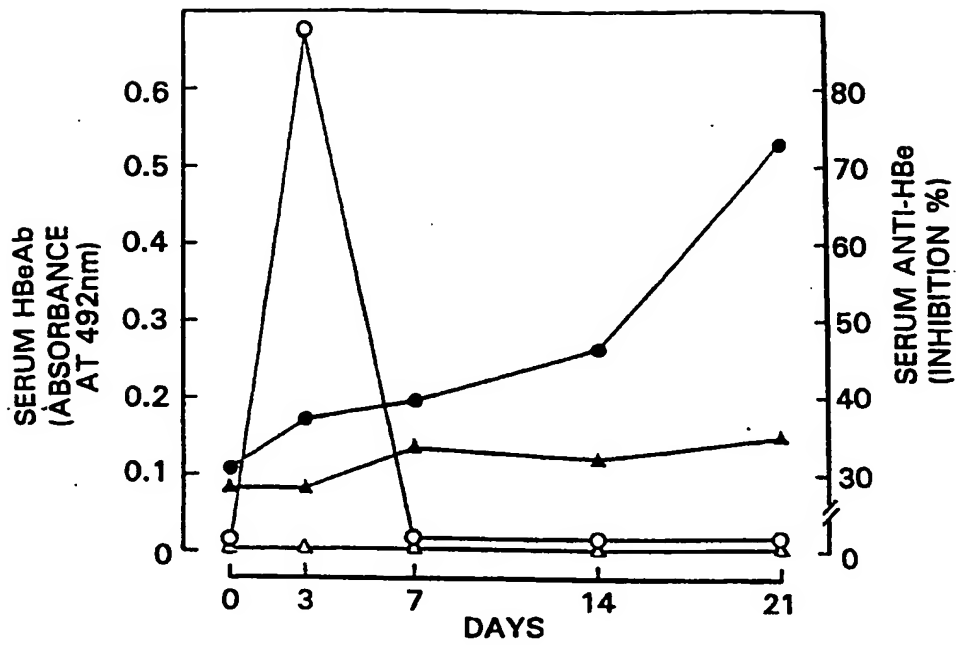
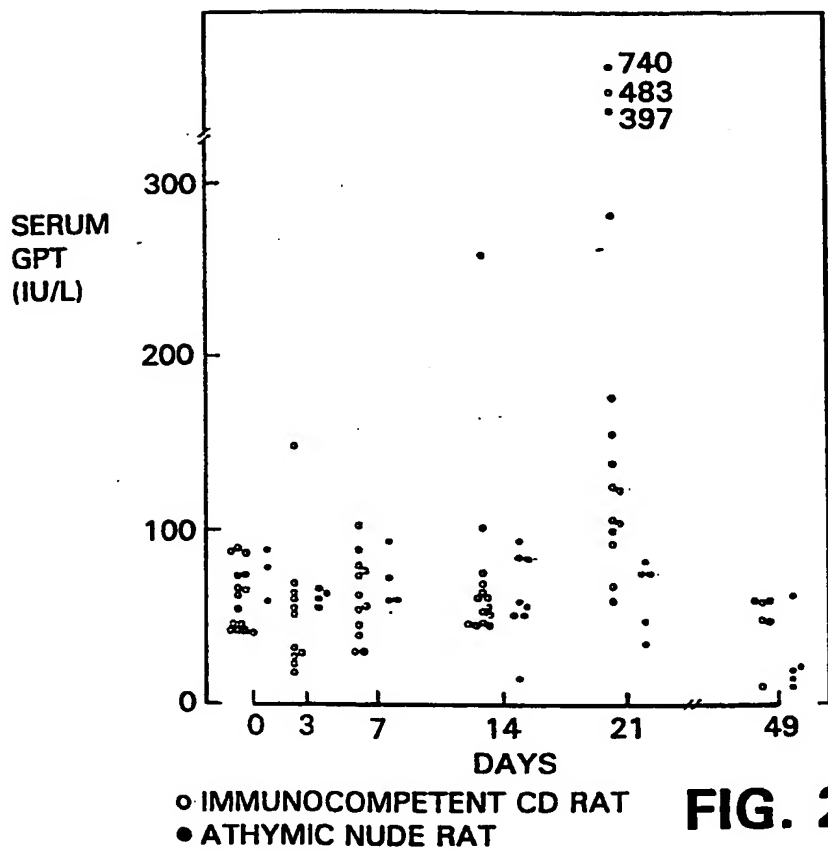


FIG. 1



○ IMMUNOCOMPETENT CD RAT
● ATHYMIC NUDE RAT

FIG. 2

SUBSTITUTE SHEET (RULE 26)

2/9

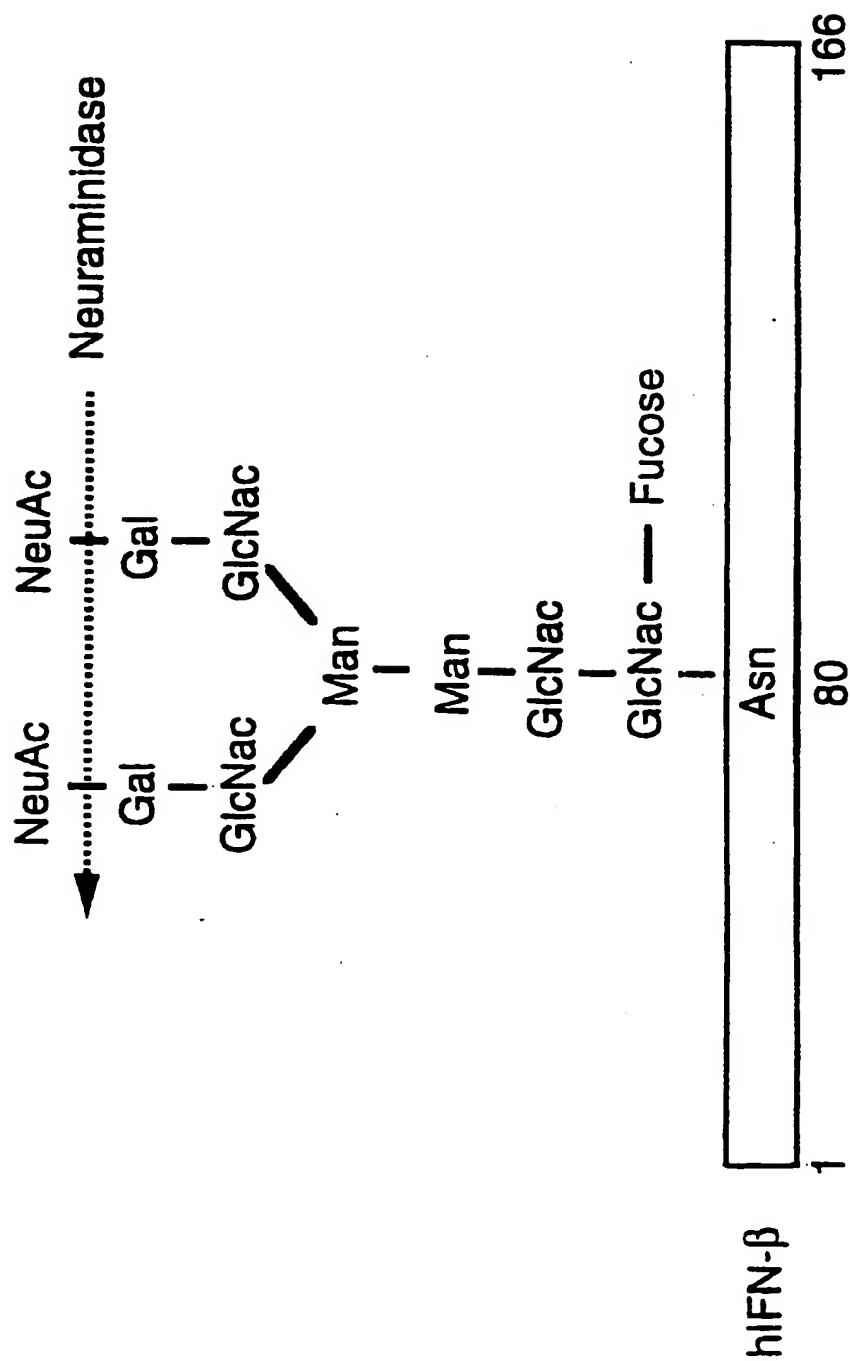


FIG. 3

3/9

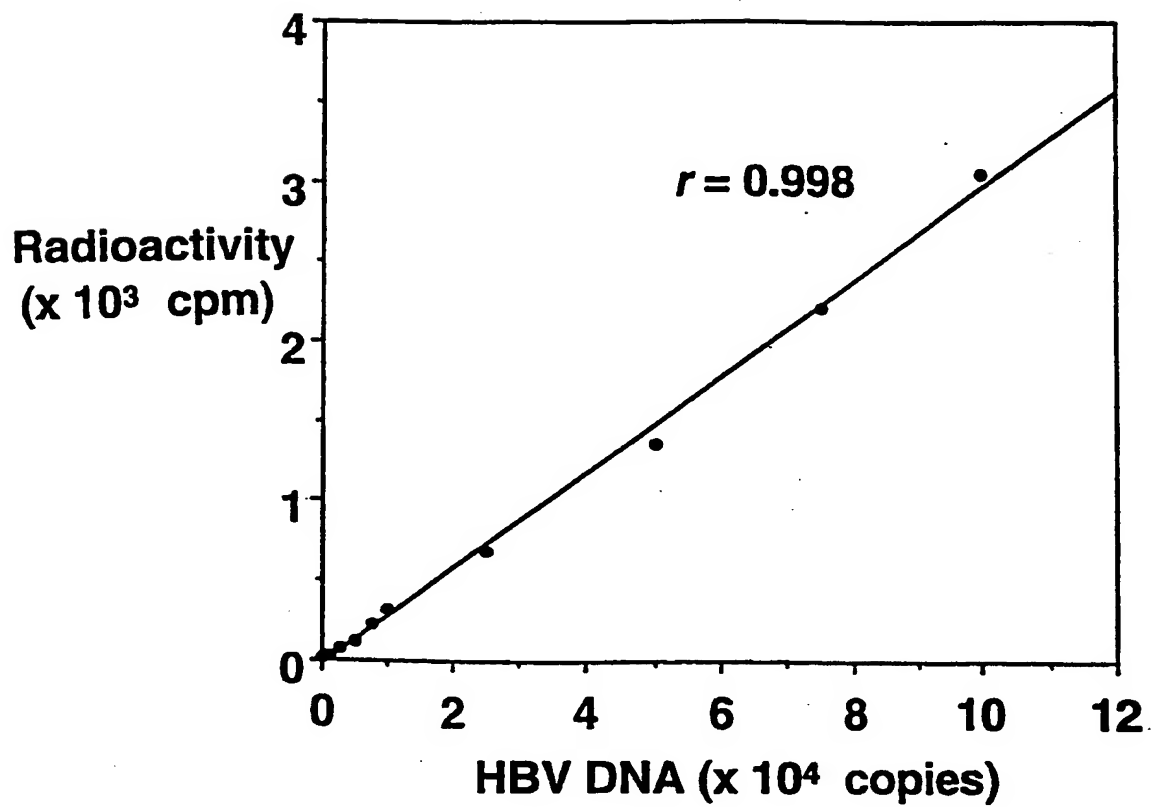
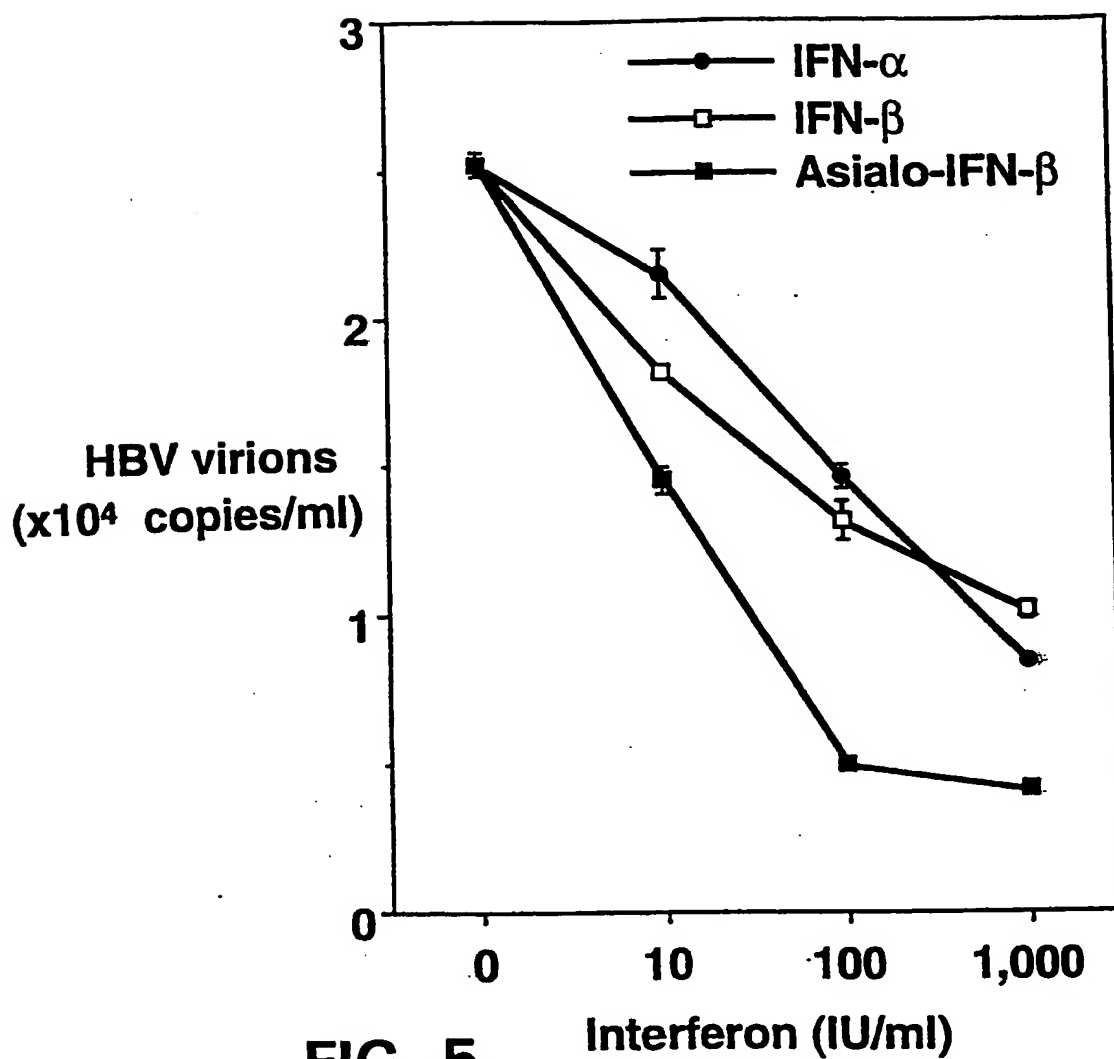
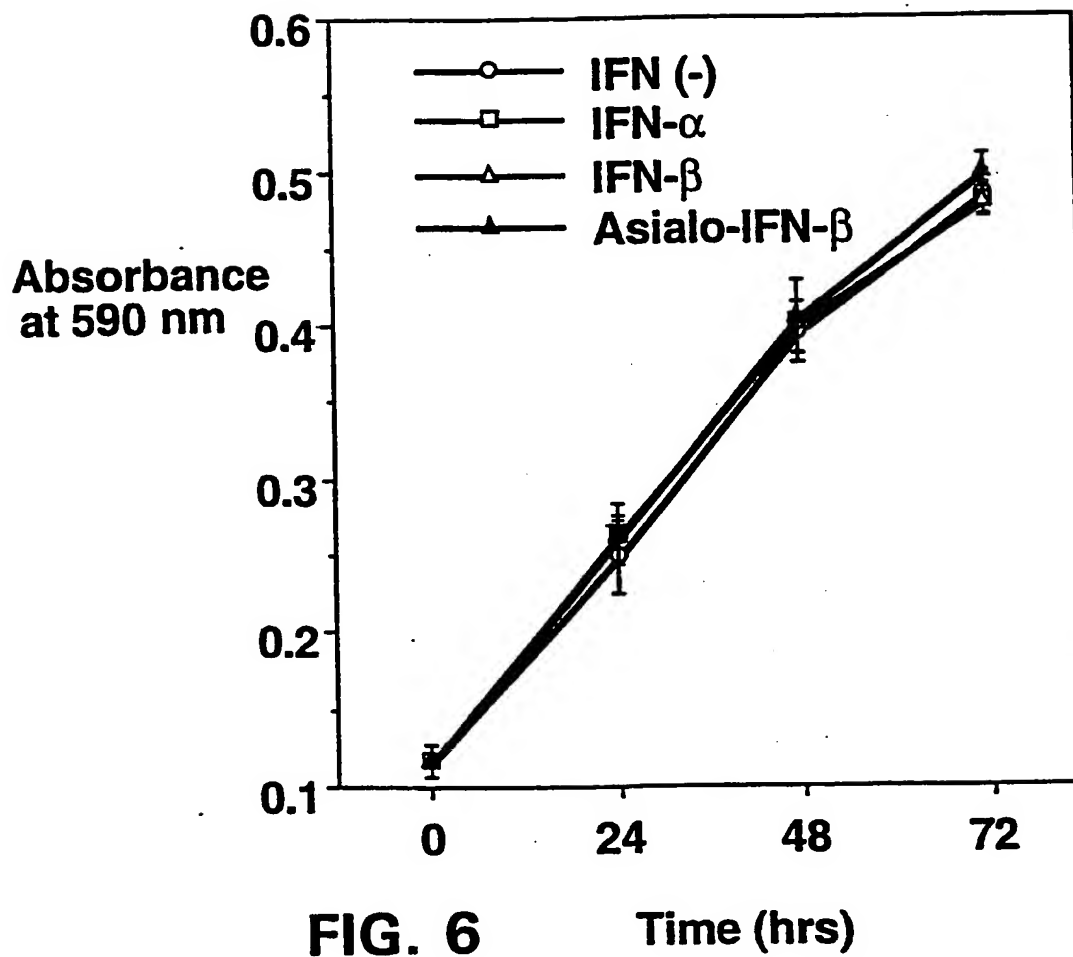


FIG. 4

4/9

**FIG. 5**

5/9

**FIG. 6** Time (hrs)

6/9

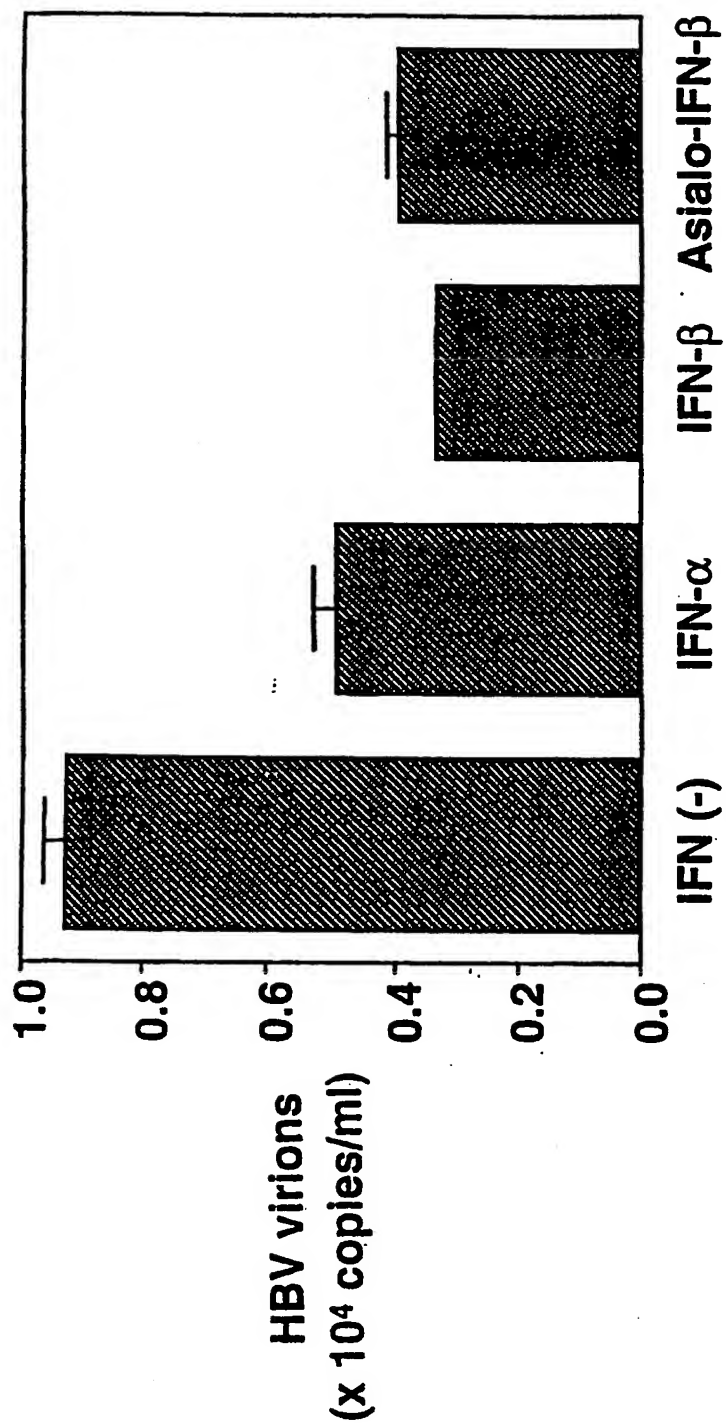


FIG. 7

7/9

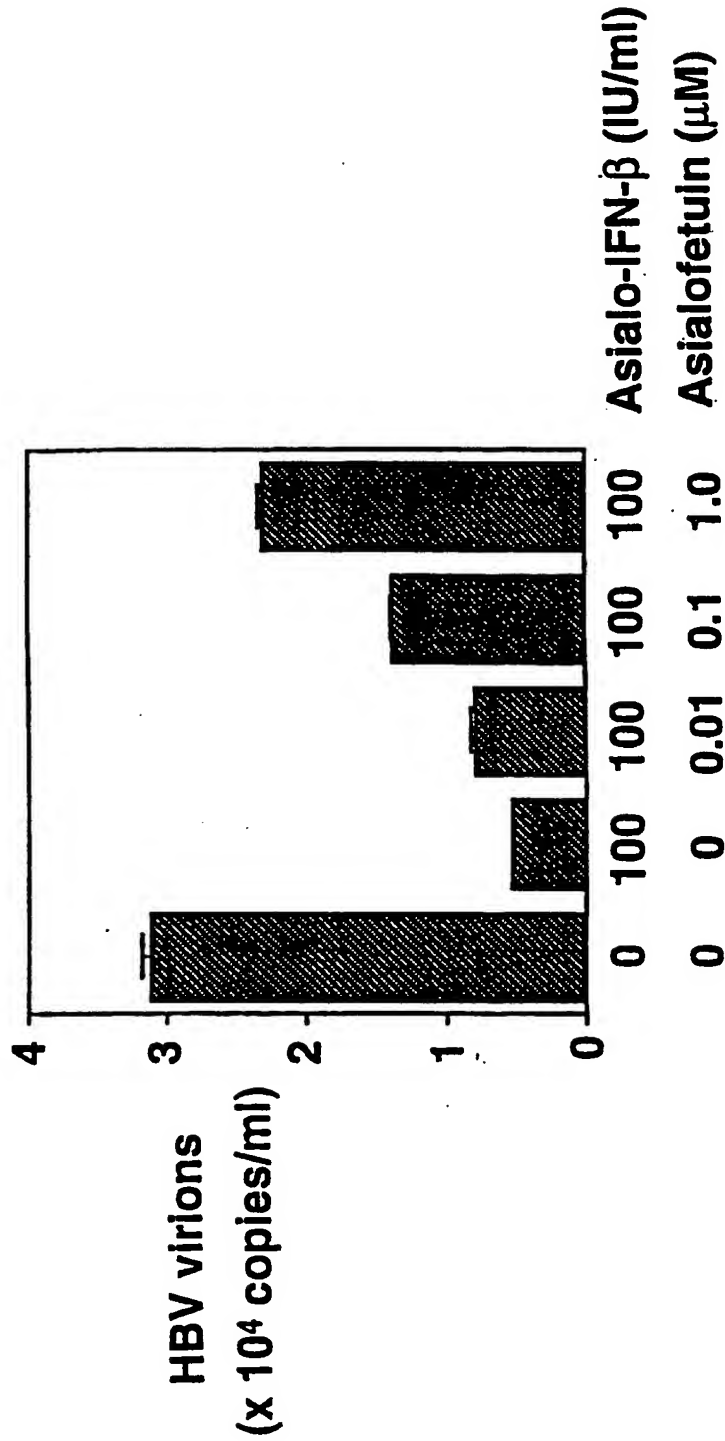


FIG. 8

8/9

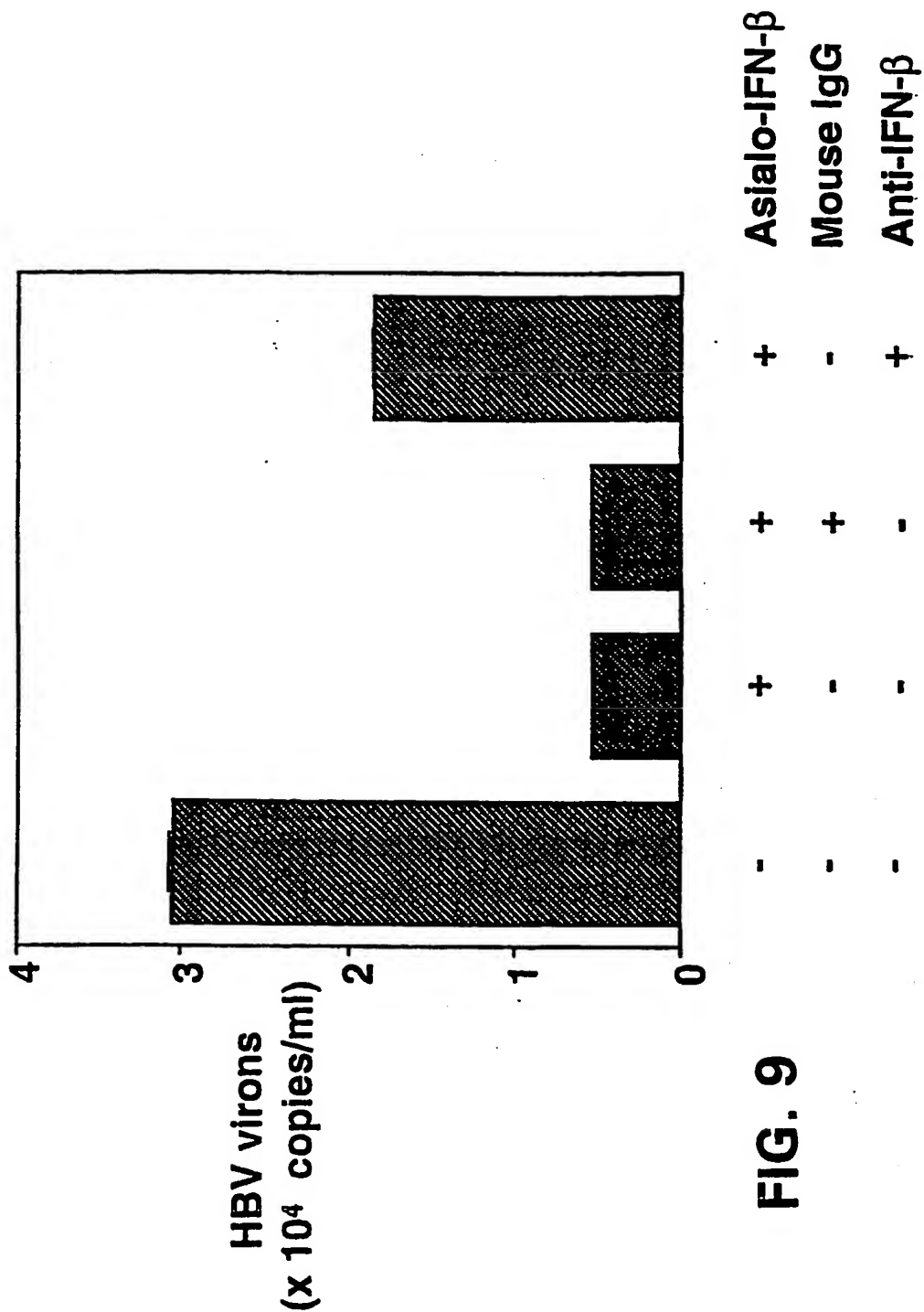


FIG. 9

9/9

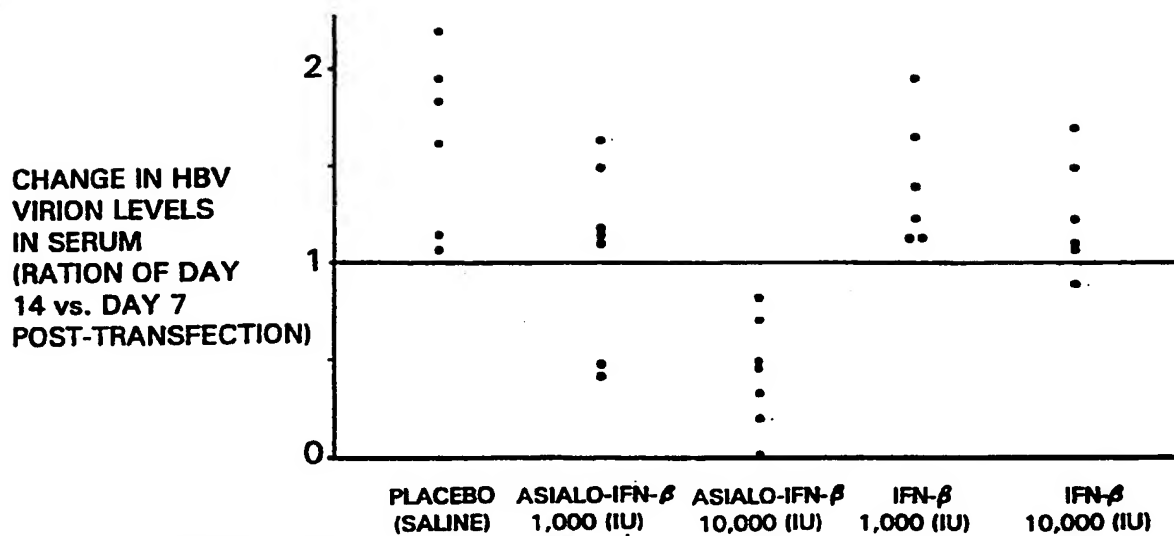


FIG. 10

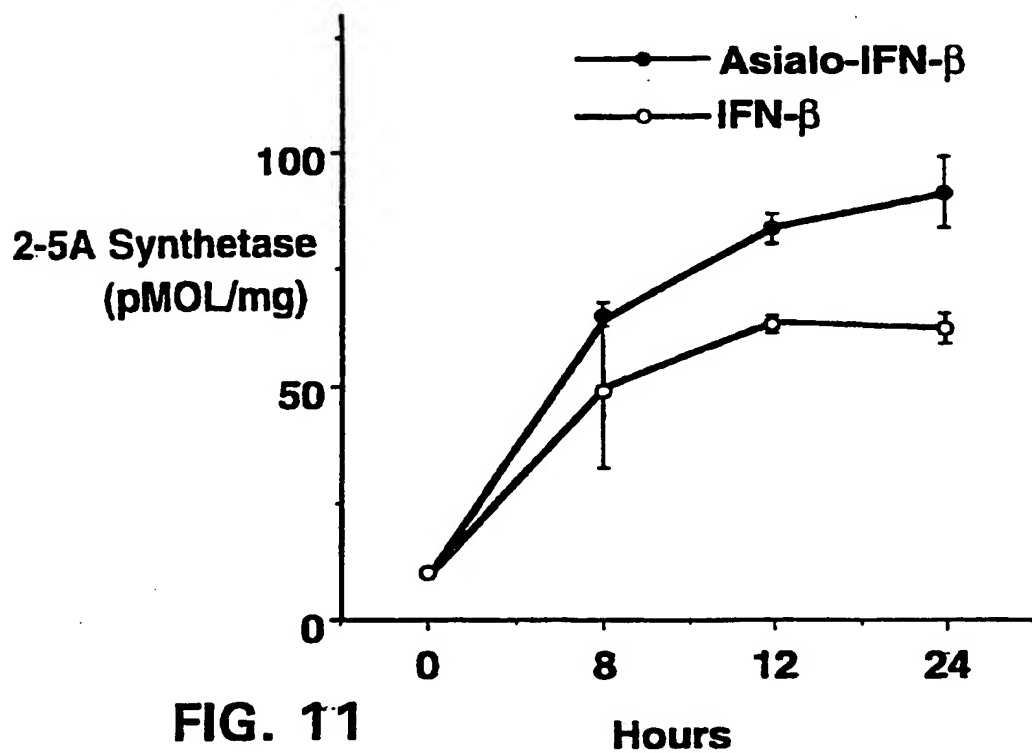


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/11225

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/00; C07K 14/00

US CL :514/2, 8; 530/350, 395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8; 530/350, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST CAPLUS BIOSIS MEDLINE EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,346,696 A (KIM et al) 13 September 1994 (13/09/94) see entire document.	1-27
X	WO 92/22310 A1 (KIM et al) 23 December 1992 (23-12-92) see entire document.	1-27
Y	ISHIHARA et al. Specific Uptake of Asialofeutin-tacked Liposomes Encapsulating Interferon-gamma by Human Hepatoma Cells and its Inhibitory Effect on Hepatitis B Virus Replication. Biochemical and Biophysical Research Communications. 31 January 1991, Vol. 174, No. 2, pages 839-845, see entire document.	1-27

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 AUGUST 2000

Date of mailing of the international search report

22 SEP 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JOSEPH F. MURPHY

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11225

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DRAGSTEN et al. Drug delivery using vesicles targeted to the hepatic asialoglycoprotein receptor. Biochimica et Biophysica Acta. 07 December 1987. Vol. 926, pages 270-279, see entire document.	1-27